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MICs of Selected Antibiotics for *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides* from a Range of Clinical and Environmental Sources as Determined by the Etest

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This paper presents Etest determinations of MICs of selected antimicrobial agents for 76 isolates of *Bacillus anthracis* chosen for their diverse histories and 67, 12, and 4 cultures, respectively, of its close relatives *B. cereus*, *B. thuringiensis*, and *B. mycoides* derived from a range of clinical and environmental sources. NCCLS breakpoints are now available for *B. anthracis* and ciprofloxacin, penicillin, and tetracycline; based on these breakpoints, the *B. anthracis* isolates were all fully susceptible to ciprofloxacin and tetracycline, and all except four cultures, three of which had a known history of penicillin resistance and were thought to originate from the same original parent, were susceptible to penicillin. Based on NCCLS interpretive standards for gram-positive and/or aerobic bacteria, all cultures were susceptible to amoxicillin-clavulanic acid and gentamicin and 99% (one with intermediate sensitivity) of cultures were susceptible to vancomycin. No group trends were apparent among the different categories of *B. cereus* (isolates from food poisoning incidents and nongastrointestinal infections and food and environmental specimens not associated with illness). Differences between *B. anthracis* and the other species were as expected for amoxicillin and penicillin, with all *B. anthracis* cultures, apart from the four referred to above, being susceptible versus high proportions of resistant isolates for the other three species. Four of the *B. cereus* and one of the *B. thuringiensis* cultures were resistant to tetracycline and a further six *B. cereus* and one *B. thuringiensis* cultures fell into the intermediate category. There was a slightly higher resistance to azithromycin among the *B. anthracis* strains than for the other species. The proportion of *B. anthracis* strains fully susceptible to erythromycin was also substantially lower than for the other species, although just a single *B. cereus* strain was fully resistant. The Etest compared favorably with agar dilution in a subsidiary test set up to test the readings, and it compared with other published studies utilizing a variety of test methods.

Little interest was shown in antimicrobial susceptibility profiles of *Bacillus* species until very recently. This was due to a combination of reasons: the low recognition of the ability of *Bacillus* species other than *Bacillus anthracis* to cause infections; the increasing rarity of human anthrax in industrialized, developed countries as a result of effective control programs over the past half century; and the high susceptibility of *B. anthracis* to penicillin coupled with the extreme rarity of reports of penicillin resistance. In developing countries where anthrax is endemic, penicillin has always been the drug of choice because of its reliability, low cost, and ready availability.

Concerns about bioaggression around the time of the 1991 Gulf War resulted in some examination of the effectiveness of more modern antimicrobials both in vitro (10, 22) and in vivo in animal models (13, 17, 20). The "anthrax letter" events of October and November 2001 in the United States further stimulated interest in antimicrobial therapy for anthrax and some debate on the appropriate therapies for different categories of patient infection (5, 6, 15, 16), with further in vitro susceptibility tests being carried out (4, 9, 12, 26). Unrelated to bioaggression, but also relevant, is the recent paper of Kadanali et al. (18) on the treatment of pregnant patients.

This study reported here, which had commenced before the anthrax letter events, was initiated to apply the Etest to as diverse a range of *B. anthracis* isolates as possible together with a set of its close relatives *B. cereus*, *B. thuringiensis*, and *B. mycoides* isolated from a range of clinical and environmental sources. The primary purpose of the study was to determine the susceptibilities of these species to a set of antibiotics selected to have the greatest guidance value to clinicians encountering anthrax, *B. cereus*, and possibly *B thuringiensis* infections in humans (*B. mycoides* has not been associated with infections). The generation of comparative susceptibility and resistance data on the members of the informally defined "*B. cereus* group" for academic purposes was the secondary aim of the work.

MATERIALS AND METHODS

Isolates. The *B. anthracis* isolates included 24 cultures (Table 1), kindly supplied by Martin Hugh-Jones and Pamala Coker, School of Veterinary Medicine, Louisiana State University, which represented all but one of the amplified fragment length polymorphism (AFLP) genotype clusters of *B. anthracis* (19). A further 52 isolates from the culture collection of the Centre for Applied Micro-

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TABLE 1. Histories of *B. anthracis* isolates included in this study (n = 76)

ID	Earlier ID and history[a]
LSU34	Genotype 57, ASC 274; bovine, China, ca. 1990 (AFLP cluster A3b)
LSU39	Genotype 55, ASC 383, outbreak in cattle, Australia, 1994 (AFLP cluster A3a)
LSU62	Genotype 15; bovine isolate, Poland, 1962 (AFLP cluster Ala)
LSU102	Genotype 85; pig isolate, Mozambique, 1944 (AFLP cluster B2)
LSU149	Genotype 23; human isolate, Turkey, 1991 (AFLP cluster A1b)
LSU158	Genotype 30; bovine isolate, Zambia, 1992 (AFLP cluster A3a)
LSU174	Genotype 3; bovine isolate, Canada, 1974 (AFLP cluster A1a)
LSU188	Genotype 35; zebra, Etosha National Park, Namibia, 1993 (AFLP cluster A3a)
LSU193	Genotype 10; bovine isolate, USA, 1996 (AFLP cluster A1a)
LSU248	Genotype 68; human isolate, USA, 1968 (AFLP cluster A3d)
LSU256	Genotype 41; human isolate, Turkey, 1985 (AFLP cluster A3a)
LSU264	Genotype 28; human isolate, Turkey, 1984 (AFLP cluster A1b)
LSU267	Genotype 25, V770-NPI-R (ATCC 14185); bovine origin, USA, 1951; human U.S. vaccine strain (2a) (AFLP cluster A1b)
LSU293	Genotype 20; sheep, Italy, 1994 (AFLP cluster A1a)
LSU328	Genotype 38; pig, Germany, 1971 (AFLP cluster A3a)
LSU376	Genotype 51; bovine, USA, 1939 (AFLP cluster A3a)
LSU379	Genotype 69; wool, Pakistan, 1976 (AFLP cluster A4)
LSU419	Genotype 34; human, South Korea, 1994 (AFLP cluster A3a)
LSU442	Genotype 87; kudu, Kruger National Park, South Africa, 1975 (AFLP cluster B2)
LSU462	Genotype 62, ASC 159; Ames strain reisolated from guinea pig, 1988 (AFLP cluster A3b)
LSU463	Genotype 29; sheep, Pakistan, 1978 (AFLP cluster A2)
LSU465	Genotype 80; bovine isolate, France, 1997 (AFLP cluster B1)
LSU488	Genotype 77; Vollum strain, Centre for Applied Microbiology and Research, Porton Down, UK (AFLP cluster A4)
LSU489	Genotype 45; bovine isolate, Argentina, 1980 (AFLP cluster A3a)
ASC 8	NCTC 109; shaving brush, Lister Institute, London, UK, 1920.
ASC 9	NCTC 1328; P. Fildes, 1922.
ASC 10	NCTC 2620; Hankow hide (Chinese). London, UK, 1928
ASC 14	ATCC 241
ASC 15	ATCC 938; Lederle Laboratories (9a)
ASC 16	ATCC 937; Lederle Laboratories (9a)
ASC 17	ATCC 944
ASC 32	Pen^r; blood culture, fatal human case, 30 Dec 1974; presumed source, bonemeal used in garden (31a)
ASC 33	C164G; human, from skin lesion, 1976; presumed source, hides
ASC 34	C149G; human, from skin lesion, 1976; presumed source, imported wool
ASC 36	C129G; human, skin lesion, 1975; presumed source, hides
ASC 38	C73G; human, from CSF of fatal case, 1974; presumed source, bonemeal
ASC 39	C165G; human, from skin lesion, 1976; presumed source, infected animal
ASC 40	Soil, Taunton, UK, 1978
ASC 42	Bovine case, Denmark
ASC 50	Z1; human isolate, 1982, from the tail end of large Zimbabwe outbreak
ASC 54	Z6, Gamma phage resistant; human isolate, 1982, from the tail end of large Zimbabwe outbreak
ASC 58	Dead elephant, Etosha National Park, 17 March 1983 (33b)
ASC 65	Chronically secreted in bovine milk, Brazil, ca. 1980; uncharacteristic colony morphology
ASC 66	From final effluent, sewage treatment works, UK, 1987
ASC 68	Ames strain
ASC 69	New Hampshire, human pulmonary anthrax, 1957 (30a)
ASC 70	Pen^r; believed to be identical to ASC 32
ASC 80	Tannery dump, UK, 11 August 1988
ASC 119	BD/WT, NSW, Australia, 1989
ASC 134	Cape Buffalo, South Luangwa National Park, Zambia, 4 August 1989
ASC 149	Gamma phage resistant; blue wildebeest, Etosha National Park, Namibia 1988
ASC 179	Laboratory demolition site, UK, 1990
ASC 182	Pasteur strain, via University of Massachusetts
ASC 183	Pen^r; pXO $1^-/2^+$; cured derivative of ASC 32, 1990
ASC 188	Cow, outbreak on sewage farm, UK, 1990
ASC 194	Elephant, 25 April 1991, Etosha National Park, Namibia
ASC 206	RNL 437; Kruger National Park, South Africa, no further history, ca. 1990
ASC 230	Soil samples from burial site of bovine 50 years previously, 10 Sept 1991, UK (33a)
ASC 259	Cow, Zambia, 1992
ASC 266	Plaster and dusty hair and wool, St. Pancras railway station, London, UK, 1992
ASC 272	Animal fur, Xingjiang Province, China, ca. 1990
ASC 319	Bovine, Scotland, 1993
ASC 324	Garden bonemeal, UK, 1993
ASC 356	Blue wildebeest, Etosha National Park, Namibia, 1993
ASC 375	Sanitary Technical Institute; Russian vaccine strain
ASC 391	Bovine dead of anthrax, Isle of Wight, UK, 1994
ASC 394	Ames reisolate; guinea pig which died of anthrax after cessation of ciprofloxacin treatment
ASC 395	Vollum reisolate; guinea pig which died of anthrax after cessation of doxycycline treatment
ASC 396	Ames reisolate; culled guinea pig 18 days after cessation of ciprofloxacin therapy
ASC 397	Vollum reisolate; lungs of culled guinea pig 18 days after cessation of ciprofloxacin therapy
ASC 398	Ames reisolate; guinea pig which died of anthrax after cessation of doxycycline treatment
ASC 399	Ames reisolate; lungs of culled guinea pig 27 days after doxycycline therapy
ASC 403	Cutaneous lesion (human), 25 Aug 1995 (3a)

Continued on following page

TABLE 1—*Continued*

ID	Earlier ID and history[a]
BDRD Ames	Ames strain via different route from ASC 68
IITRI B1	History not known
IITRI B2	History not known
IITRI B3	History not known

[a] USA, United States; UK, United Kingdom; NSW, New South Wales; CSF, cerebral spinal fluid.

biology and Research, Health Protection Agency, Porton Down, Salisbury, United Kingdom, were chosen on the basis of being as diverse as possible in terms of (i) geographic source, (ii) year of isolation, (iii) type of source (human, animal, or environmental), (iv) known or likely laboratory manipulation (frequent passage or deliberate curing of one or both plasmids), and (v) known unusual characteristics, particularly penicillin or phage resistance. Of the 76 total cultures included, 59 were believed to be unrelated epidemiologically. All manipulations of *B. anthracis* were carried out in class 2 microbiological safety cabinets within the Biological Defense Research Directorate (BDRD) biosafety level 3 (BSL3) facility under strict safety protocols and meeting all the requirements of DHHS 42 CFR 73 (12a).

In addition, a selection of closely related nonanthrax *Bacillus* species acquired from the Food Safety and Microbiology Laboratory, Central Public Health Laboratory, London, United Kingdom, and Niall Logan, Department of Biological Sciences, Glasgow Caledonian University, Glasgow, United Kingdom, were also included in the study (Table 2). These cultures were chosen to encompass isolates implicated in nongastrointestinal infections and food poisoning incidents and simple environmental isolates.

Staphylococcus aureus ATCC 29213 was included as a test control.

Etests. Cultures were grown on Mueller-Hinton agar (MHA) overnight at 36°C ± 1°C. For each test, growth from approximately five colonies was emulsified in 1 ml of sterile saline, and this was used to make a suspension, again in sterile saline, with turbidity equivalent to a 0.5 McFarland standard. This turbidity level was established with 18 of the cultures, representative of the different species included in the study, to fall within the range of 3×10^6 to 20×10^6 CFU/ml, compared against 1×10^8 to 3×10^8 CFU/ml for *S. aureus* ATCC 29213. Sterile swabs dipped into this suspension and squeezed against the side of the suspension tube to remove excess fluid were streaked across predried MHA plates (90 mm), three times for each plate, with the plate rotated approximately 90° between each streaking. After approximately 10 to 15 min, to allow absorption of excess moisture into the agar, two Etest strips (AB Biodisk North America Inc., N.J.) per plate in opposing directions were placed on either side of each plate.

The plates were incubated at 36°C ± 1°C for 18 to 20 h, and the MICs were read according to the manufacturer's instructions.

Inoculum size and incubation time and temperature. The Etest manufacturer's specifications for inoculum size for aerobes is based on bringing the culture to a turbidity equivalent to a 0.5 McFarland standard. *Bacillus* species, being comprised of large rods, had lower counts at this turbidity level than did smaller bacteria such as the gram-positive cocci or the *Enterobacteriaceae*. Being rapid growers, producing large colonies by 16 to 24 h, the lawns on the plates of *Bacillus* species also have different properties from lawns of more frequently encountered pathogenic aerobes. Additionally, the manufacturer specifies an incubation temperature of 35°C. This temperature is not necessarily a convenient specification for a laboratory incubator or the optimum incubation temperature for *Bacillus* species. Tests were therefore set up to assess the influence of inoculum size, temperature, and time of incubation. Fourteen of the strains, three clinical (F77/1589, F78/667, and F95/8201), two food poisoning (F72/4810 and F73/4433), and two environmental (F99/5739 and F00/3016) isolates of *B. cereus* and two *B. thuringiensis* (B1143 and F98/5750) and five *B. anthracis* (Ames, ASC 32, LSU102, LSU248, and LSU293) strains, chosen from the main set of tests as being representative of diverse susceptibility readings with the antibiotics, were retested by using inocula with turbidities of 0.5, 2.0, and 4.0 and with the test plates incubated at carefully controlled temperatures of 30, 35, and 37°C, followed by readings at 16 and 24 h. *S. aureus* ATCC 29213 was again included for comparison. The readings were analyzed statistically by the unpaired Student's *t* test.

Agar dilution MIC tests. For purposes of direct comparison of the Etest results with a conventional procedure, the MICs for 10 of the *B. anthracis* strains, 15 of the *B. cereus* strains (5 food and environmental, 5 food poisoning, and 5 nongastrointestinal infection isolates), 5 of the *B. thuringiensis* strains, and the 4 *B. mycoides* strains, together with *S. aureus* ATCC 29213, were tested by using an agar dilution method described previously (22). Each antibiotic was diluted and incorporated into 100 ml of MHA to create a series of plates (150 mm) ranging from 64 to 0.015 mg/liter. In practice, this involved adding 5 ml of 20× solutions in sterile deionized water, prewarmed to 44°C, to 95 ml of sterilized MHA also at 44°C prior to the solutions being poured onto the plates. Because preliminary trials established no difference in results between the use of 1:10 and 1:50 dilutions of these suspensions, the final inoculum chosen was 5 μl of a 1:25 dilution of the 0.5 McFarland standard suspensions, established by plate counts to be equivalent to approximately 1,000 CFU. Duplicate 5-μl drops of the diluted culture suspensions were placed onto each plate by using a location grid.

RESULTS

The Etest results are summarized in Table 3. Where NCCLS breakpoints have been established for *B. anthracis* (ciprofloxacin, penicillin, and tetracycline) (27), the interpretation of susceptibility has been based on those breakpoints. For the other antibiotics in the case of *B. anthracis* and for all the antibiotics in the case of the nonanthrax *Bacillus* species, the susceptibility and resistance judgments are based on NCCLS interpretive standards for gram-positive and/or aerobic bacteria as given in the Etest manufacturer's product inserts.

Differences between *B. anthracis* and the other species were as expected for penicillin and amoxicillin-clavulanic acid (97% of 74 and 100% of 45 *B. anthracis* cultures, respectively, were sensitive compared with high proportions of resistant isolates in the other species). The presence of some isolates of *B. cereus* and *B. thuringiensis* that were resistant to tetracycline was also probably to be expected. The less anticipated result was a slightly lower susceptibility to azithromycin among the *B. anthracis* strains (only 26% fully susceptible and 10% resistant) than the other species (≥84% fully susceptible and none entirely resistant). The proportion of *B. anthracis* strains that were fully susceptible to erythromycin (15%) was substantially lower than that with the other species (≥78%), although only one strain of *B. cereus* was fully resistant.

An analysis of results for the different categories of *B. cereus* (isolates from food poisoning incidents and nongastrointestinal infections and food and environmental specimens not associated with illness) did not reveal any group trends (details not presented). Apparent species differences between *B. cereus*, *B. thuringiensis*, and *B. mycoides* with cefotaxime (29% of *B. cereus* isolates were susceptible versus none of the *B. thuringiensis* and *B. mycoides* isolates) may simply reflect the relatively small numbers of *B. thuringiensis* and *B. mycoides* strains included.

In relation to penicillin sensitivity, ASC 32, ASC 70, and ASC 183 were counted as a single strain so as not to distort the percentage of the total of strains that were penicillin resistant. The unusual resistance of ASC 32 and ASC 70 to penicillin has already been noted (22). A single isolate, LSU 62, was fully susceptible and a second isolate, ASC 65, had intermediate susceptibility to cefotaxime. This may be a good strain marker for these cultures, which in fact have other slightly unusual characteristics; LSU 62 is the only strain of *B. anthracis* that we

TABLE 2. Histories of the *B. cereus*, *B. thuringiensis*,[a] and *B. mycoides*[b] strains included in this study

Circumstance of isolation	*B. cereus* isolate; source
Nongastrointestinal infection	F77/1589[c]; bovine mastitis, serotype 12
	F77/2809A; infant born very edematous, serotype 6
	F78/660; facial burns, cellulitis developed, serotype 20
	F78/667; gangrenous postoperative wound, serotype 2
	F78/928; septic amputation stump, not typeable
	F78/968; postoperative wound, severe infection, serotype 21
	F95/2410; gangrene, cellulitis, serotype 26
	F95/6445; infected insect bite, not typeable
	F95/8201; endocarditis, not typeable
	F95/9251; pacemaker wire site infection, serotype 16
	F95/9896; ascites, not typeable
	F97/5782; eye, vitreous, serotype 4
	F98/2556; leg ulcer swab, serotype 6
	F98/2752; infected surgical wound, serotype 2
	F98/5462; sputum, cystic fibrosis, serotype 17B
	F98/5758; sticky eye, newborn, serotype C
	F98/5801; infected leg, serotype 20
	F99/3177; wound swab, serotype 17
	F00/3037; leg swab, serotype 17, *V*
	F00/3086; neutropenia in child, serotype 3
	G9241; fatal pneumonia
Food poisoning	F72/4810; from cooked rice, associated with vomiting, serotype 1
	F73/4430 (strain 4ac); from pea soup associated with diarrhea
	F73/4433; from meat loaf associated with diarrhea, serotype 2
	F75/4552; vomit isolate, serotype 3
	F95/5060 fecal isolate, diarrhea and vomiting, serotype 8
	F95/5156; fecal isolate, serotype 1
	F96/4966; vomit isolate, serotype 1
	F96/4977; fecal isolate associated with food poisoning, serotype 22
	F97/1154; vomit isolate, serotype 29
	F97/4284; fecal isolate associated with food poisoning, serotype 14
	F97/4144; vomit isolate, serotype AA
	F98/3368; fecal isolate associated with food poisoning, serotype 1
	F98/4499; fecal isolate, diarrhea and vomiting, serotype 1
	F99/502; from rice pudding associated with food poisoning, serotype AA
	F99/3957; fecal isolate associated with food poisoning, serotype 1
	99/3959; fecal isolate associated with food poisoning, serotype 20
Food and environment and other sources	F74/2532B; raw rice, nontoxigenic, not typeable
	F95/3027; orthopedic-related area, not typeable
	F95/3030; orthopedic-related area, serotype 20
	F95/3032; orthopedic-related area, serotype 24
	F95/3780; rice survey, serotype 5
	F95/8199; settle plates, special care baby unit, not typeable
	F95/8200; settle plates, special care baby unit, serotype 6
	F95/9125; washing machine door in hospital, serotype 29
	F95/9130; water rack, hospital isolate, serotype 1
	F98/2620; industrial fermenter, antibiotic production, serotype 1
	F98/2658; A1 Hakkam isolate, Iraq, not typeable[d]
	F98/3850; dishwasher in hospital, serotoype 21
	F98/3851; dishwasher in hospital, serotype 1
	F98/5379; laundry environment, serotype 29
	F99/1129; malt extract, not typeable
	F99/1695; orthopedic theater, not typeable
	F99/2864; London, United Kingdom, bombing, not typeable
	F99/4750; rice survey, not typeable
	F99/4860; milk
	F99/5739; top of locker, hospital, theater environment, serotype 20
	F99/5931; food-water-environment survey isolate, serotype 20
	F99/5941; food-water-environment survey isolate
	F99/6253; spice survey, serotype 29
	F00/2507; food-water environment survey isolate, Tandoori chicken, serotype A
	F00/2782; survey, serotype 11
	F00/3016; milk
	F00/3020; milk
	F00/3096; egg fried rice
	F00/3130; milk, serotype 20
	ATCC 10987

[a] The 12 *B. thuringiensis* strains were from culture collections, with identities as follows: 150 Dulmage 39, 152 Dulmage 3, 166 Dulmage 137, 1139 "var darmstadiensis," 1143 "var israelensis," B157 Dulmage 5 "B. sotto," B164 Dulmage 10 "B. subtoxicus," B162 Dulmage 29, B1140 "var toumanoffi," de Barjac, F98/5750, F99/4759, F99/2934.

[b] The four *B. mycoides* strains were from culture collections, with identities as follows: F95/1883, F96/3308, DSM 299 (1976), NRS 936 (1978) "B. praussnitzi."

[c] Food Safety and Microbiology Laboratory number.

[d] Considered by some workers to be *B. thuringiensis* but identified in the Food Safety and Microbiology Laboratory as *B. cereus.*

have encountered which will not grow on the well-established polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar used for selective isolation of *B. anthracis* from environmental samples, and ASC 65 produces colonies resembling those of *Enterobacteriaceae*. The inhibitory component of PLET for LSU 62 was shown not to be polymyxin.

ASC 32, ASC 70, and ASC 183 exhibited complete resistance with no zones of clearing. One of the strains (LSU 102) reported to be penicillin resistant by Coker et al. (9) did exhibit a resistant subpopulation with colonies present in the ellipse. This strain was therefore deemed resistant to penicillin. In this study, resistance was not noted with the other two strains Coker et al. recorded as being resistant (LSU 248 and LSU 293). All three LSU strains were included in the subsidiary study on the effect of inoculation size and incubation temperature and time. While the four resistant cultures had elevated MICs of amoxicillin and clavulanic acid compared with the others, they still fell well within the susceptible category as it is presently defined.

None of the Ames or Vollum strain reisolates ASC 394 to ASC

TABLE 3. Etest results for all *Bacillus* species tested

Antibiotic	Species	No. of isolates	MIC (μg/ml)			Breakpoints[a]		Interpretation (%)[b]		
			Range	50%	90%	S (≤)	R (≥)	S	I	R
Amoxicillin-clavulanic acid	*B. anthracis*	48[c]	0.016–0.5	0.032[g]	0.047	4	8	100		
	B. cereus	67	0.5–64	8	12			22	18	60
	B. thuringiensis	12	4–96	12	24			8	8	84
	B. mycoides	4	8–24	8	24					100
	S. aureus 29213		0.4 (0.25–1)[d]							
Azithromycin	*B. anthracis*	73	1–12	3	6	2[a]	8[a]	26	64	10
	B. cereus	67	0.094–6	0.38	3			84	16	
	B. thuringiensis	12	0.094–3	0.19	3			84	16	
	B. mycoides	4	0.19–0.38	0.19	0.38			100		
	S. aureus 29213		1.5 (1.5–4)[d]							
Cefotaxime	*B. anthracis*	76	3–>32	>32	>32	8[a]	64[a]	1	1	98[e]
	B. cereus	67	0.1–>32	>32	>32			29		71[e]
	B. thuringiensis	12	>32	>32	>32					100[e]
	B. mycoides	4	>32	>32	>32					100[e]
	S. aureus 29213		2 (1.5–2)[d]							
Ciprofloxacin	*B. anthracis*	76	0.032–0.094	0.064	0.094	0.5[f]		100		
	B. cereus	67	0.047–0.5	0.19	0.25	1[a]	4[a]	100		
	B. thuringiensis	12	0.094–0.19	0.125	0.19			100		
	B. mycoides	4	0.125–0.25	0.125	0.25			100		
	S. aureus 29213		0.5 (0.25–0.5)[d]							
Erythromycin	*B. anthracis*	69	0.5–4	1	1.5	0.5[a]	8[a]	15	85	
	B. cereus	67	0.032–3	0.064	1.5			78	21	1
	B. thuringiensis	12	0.032–1	0.094	1			84	16	
	B. mycoides	5	0.064–0.38	0.125	0.25			100		
	S. aureus 29213		0.38 (0.25–0.5)[d]							
Gentamicin	*B. anthracis*	75	0.064–0.5	0.25	0.38	4[a]	16[a]	100		
	B. cereus	67	0.094–0.75	0.38	0.75			100		
	B. thuringiensis	12	0.047–0.5	0.19	0.5			100		
	B. mycoides	5	0.19–0.38	0.25	0.25			100		
	S. aureus 29213		0.8 (0.5–1.5)[d]							
Penicillin	*B. anthracis*	74[c]	<0.016–≥32	<0.016	0.023	0.12[a,f]	0.25[a,f]	97		3
	B. cereus	67	0.012–>32	>32	>32			1		99
	B. thuringiensis	12	>32	>32	>32					100
	B. mycoides	4	>32	>32	>32					100
	S. aureus 29213		0.4 (0.25–0.38)[d]							
Tetracycline	*B. anthracis*	71	0.016–0.094	0.023	0.032	1		100		
	B. cereus	67	0.05–32	1	6	4[a]	16[a]	84	9	6
	B. thuringiensis	12	0.5–24	2	6			84	8	8
	B. mycoides	4	0.125–2	0.5	2			100		
	S. aureus 29213		0.17 (0.094–0.38)[d]							
Vancomycin	*B. anthracis*	74	0.75–5	2	3	4[a]	32[a]	99	1	
	B. cereus	67	1–16	3	6			85	15	
	B. thuringiensis	12	0.75–4	2	4			100		
	B. mycoides	4	1.5–4	2				100		
	S. aureus 29213		1.8 (1.5–2)[d]							

[a] NCCLS MIC interpretive standards for gram-positive and/or aerobic bacteria (NCCLS documents M100-S6, M7-A3, and M11-A3 [1995]; M100-S7, M7-A4, and M11-A3 [1997]; M100-S8 and M7-A4 [1998], and M100-S9 and M7-MIC [1999]), as given in the manufacturer's insert.

[b] S, susceptible; I, intermediate; R, resistant.

[c] Penicillin-resistant cultures ASC 32, ASC 70, and ASC 183 are treated here as one strain.

[d] The values given for *S. aureus* ATCC 29213 are the means and ranges of 5 to 7 repeat tests.

[e] Resistance inferred. The highest level on the strips used was 32 μg/ml.

[f] NCCLS approved standard M100-S13 (27). No tetracycline- or ciprofloxacin-resistant strains were available for establishing the standards; only susceptible breakpoints were established for these drugs.

[g] MIC at which 50% of the isolates tested are inhibited.

TABLE 4. Comparison of MIC results by Etest and agar dilution

Antibiotic	Method[a]	MIC (μg/ml) for *Bacillus* species (no. tested)					
		B. anthracis (10)[a]			*B. cereus* (15), *B. thuringiensis* (4), and *B. mycoides* (3)		
		Range	50%	90%	Range	50%	90%
Amoxicillin-clavulanic acid	Etest	0.016–0.5	0.032	0.5	1.5–16	8	16
	AD	0.015–0.06	0.03	0.06	0.015–8	2	4
Cefotaxime	Etest	>32	>32	>32	0.1–>32	>32	>32
	AD	16–64	32	32	0.015–>64	32	>64
Ciprofloxacin	Etest	0.047–0.094	0.064	0.094	0.094–0.38	0.125	0.25
	AD	0.06	0.06	0.06	0.03–0.125	0.125	0.25
Erythromycin	Etest	0.5–4	0.75	1	0.064–6	0.25	2
	AD	0.5–2	1	2	0.5–4	0.5	4
Gentamicin	Etest	0.064–0.5	0.25	0.38	0.125–0.75	0.25	0.75
	AD	0.25–0.5	0.25	0.5	0.25–1	0.5	1
Penicillin	Etest	<0.016–>32	<0.016	0.023	1.5–>32	>32	>32
	AD	0.015–0.5	0.015	0.015	0.125–16	8	16
Tetracycline	Etest	0.016–0.032	0.023	0.032	0.05–32	0.75	16
	AD	0.015–0.06	0.015	0.03	0.015–32	0.25	4
Vancomycin	Etest	1–3	2	3	0.75–4	3	4
	AD	1–4	4	4	0.015–4	2	4

[a] AD, agar dilution.

399, from guinea pigs which died after the cessation of ciprofloxacin or doxycycline prophylaxis following infection by the inhalational route (17), had developed observable resistance.

In the tests carried out to assess the effect of inoculum size and temperature and time of incubation, no significant differences in the readings were found for any of the starting inocula (turbidity equivalents of 0.5, 2.0, and 4.0), incubation temperature (30, 35, or 37°C), or reading times (16 or 24 h) ($P = 0.16$ to 1.00 for all, except for the comparison of vancomycin tests read at 30 and 35 or 37°C, for which $P = 0.06$).

In the subsidiary tests done to compare Etests with a conventional MIC approach, although only 78% of the Etest and agar dilution MIC readings were within 1 agar dilution unit of each other (96% in the case of the tests on *B. anthracis* alone), the only disagreement in terms of judgements as to susceptibility or resistance were that *B. anthracis* and *B. cereus* cultures, which were

TABLE 5. Comparison of reports on MICs for *B. cereus*[a]

Antibiotic	Source of reference	Test method	No. of strains	MIC (μg/ml)		
				Range	50%	90%
Cefotaxime	This study	Etest	67	0.1–>32	>32	>32
	This study	Agar dilution	15	0.015–>64	16	32
	35	Microdilution	54	16–>128	32	>128
Ciprofloxacin	This study	Etest	67	0.047–0.5	0.19	0.25
	This study	Agar dilution	15	0.03–0.25	0.125	0.125
	35	Microdilution	54	≤0.25–1	≥0.25	1
Erythromycin	This study	Etest	67	0.032–3	0.064	1.5
	This study	Agar dilution	15	0.5–4	0.5	4
	28	NA	6	<0.25–0.5	NA	NA
Gentamicin	This study	Etest	67	0.094–0.75	0.38	0.75
	This study	Agar dilution	15	0.25–1	0.5	1
	28	NA	6	<0.12–0.5	NA	NA
Penicillin	This study	Etest	67	0.012–>32	>32	>32
	This study	Agar dilution	15	0.5–16	8	16
	28	NA	6	4–>8	NA	NA
Tetracycline	This study	Etest	67	0.05–32	1	6
	This study	Agar dilution	15	0.015–32	0.25	32
	28	NA	6	<0.12–4	NA	NA
Vancomycin	This study	Etest	67	1–16	3	6
	This study	Agar dilution	15	1–4	2	4
	28	NA	6	0.5–1	NA	NA
	35	Microdilution	54	≤0.25–2	2	2

[a] NA, information not available.

TABLE 6. Comparison of reports on MICs for *B. anthracis*

Antibiotic	Source or reference	Test method	No. of strains	MIC (μg/ml)			Breakpoints[a]		Interpretation (%)[b]		
				Range	50%	90%	S (≤)	R (≥)	S[e]	I	R
Amoxycillin-clavulanic acid	This study	Etest	45	0.016–0.5	0.032	0.047	4	8	100		
	This study	Agar dilution	10	0.015–0.06	0.03	0.06			100		
	4	Agar dilution	96	0.125–16	0.125	4			88.5		11.5
	10	Agar dilution	22	0.015–0.015	0.015	0.015			100		
	22	Agar dilution	70	0.03–64	0.125	0.06			99		1
Azithromycin	This study	Etest	73	1–12	3	6	2	8	26	64	10
Cefotaxime	This study	Etest	76	3–>32	>32	>32	8	64	1		99[c]
	This study	Agar dilution	10	16–64	32	32				100	
	10	Disk diffusion	22						4	14	82
	10	Agar dilution	22	8–32	32	32					
	29	Disk diffusion	44							100	
Ciprofloxacin	This study	Etest	76	0.032–0.094	0.064	0.094	≤0.5		100		
	This study	Agar dilution	10	0.06	0.06	0.06			100		
	4	Agar dilution	96	0.03–0.5	0.06	0.25			100		
	9	Etest	25	0.032–0.38	0.094	0.094			100		
	10	Agar dilution	22	0.03–0.06	0.03	0.06			100		
	10	Disk diffusion	22						100		
	12	Agar dilution	40	<0.008–0.12	0.03	0.06			100		
	Klugman et al.[f]	NCCLS methods	25	0.0625–0.125	0.0625	0.0625			100		
	22	Agar dilution	70	0.03–0.06	0.06	0.06			100		
	26	Agar dilution	65	0.03–0.12	0.06	0.06			100		
	Brooks et al.[d]	Microdilution	12	<0.25	<0.25	<0.25			100		
Erythromycin	This study	Etest	69	0.5–4	1	1.5	0.5	8	15	85	
	This study	Agar dilution	10	0.5–2	1	2			10	90	
	4	Agar dilution	96	0.5–4	1	1			95.4	4.6	
	10	Disk diffusion	22						100		
	Klugman et al.	NCCLS methods	25	0.125–4	0.5	1			NA		
	22	Agar dilution	70	0.25–1	0.5	1			NA	NA	0
	26	Agar dilution	65	0.5–1	1	1			3	97	
	29	Disk diffusion	44						100		
	Brooks et al.	Microdilution	12	0.25–4	0.5	1			8	92	
Gentamicin	This study	Etest	75	0.064–0.5	0.25	0.38	4	16	100		
	This study	Agar dilution	10	0.25–0.5	0.25	0.5			100		
	4	Agar dilution	96	0.125–0.5	0.25	0.5			100		
	10	Agar dilution	22	0.03–0.25	0.06	0.125			100		
	10	Disk diffusion	22						100		
	22	Agar dilution	70	0.06–0.5	0.125	0.25			100		
	29	Disk diffusion	44						97.8	2.2	
Penicillin	This study	Etest	74	<0.016–>32	<0.016	0.023	0.12	0.25	97		3
	This study	Agar dilution	8	0.015–0.5	0.015	0.015			—[e]		
	4	Agar dilution	96	0.125–16	0.125	8			88.5		11.5
	9	Etest	25	<0.016–0.5	0.042	0.236			88		12
	10	Agar dilution	22	0.015–0.03	0.015	0.015			100		
	10	Disk diffusion	22						100		
	12	Agar dilution	40	0.016–0.03	0.016	0.016			100		
	22	Agar dilution	70	0.015–64	0.06	0.125			99		1
	26	Agar dilution	65	<0.06–128	≤0.06	≤0.06			97		3
	29	Disk diffusion	44						84.1	15.9	
Tetracycline	This study	Etest	71	0.016–0.094	0.023	0.032	1		100		
	This study	Agar dilution	10	0.015–0.06	0.015	0.03			100		
	10	Disk diffusion	22						100		
	22	Agar dilution	70	0.6–1	0.125	0.125			100		
	26	Agar dilution	65	0.03–0.06	0.03	0.06			100		
	29	Disk diffusion	44						100		
Vancomycin	This study	Etest	74	0.75–5	2	3	4	32	99	1	
	This study	Agar dilution	10	1–4	4	4			100		
	4	Agar dilution	96	0.25–2	1	1			100		
	10	Agar dilution	22	0.25–1	1	1			95	5	
	10	Disk diffusion	22						95	5	
	26	Agar dilution	65	0.5–2	2	2			100		

[a] NCCLS MIC interpretive standards (see footnotes to Table 3). Interpretations in the cited reports predate the newly available NCCLS breakpoints for *B. anthracis* and ciprofloxacin, penicillin, and tetracycline.

[b] S, susceptible; I, intermediate; R, resistant; NA, information not available.

[c] Resistance inferred. The highest level on the strips used was 32 μg/ml.

[d] T. Brooks, P. C. B. Turnbull, and A. Maule, unpublished results.

[e] —, for penicillin, ASC 32, ASC 70, and ASC 183 resistant; remainder susceptible.

[f] K.P. Klugman, J. Frean, L. Arntzen, V. Yeldandi, and S. Bukofzer, Addendum, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. UL-20, p. 7, 2001.

seen as having intermediate susceptibility to cefotaxime by agar dilution, were fully resistant by Etests (Tables 4 and 5).

DISCUSSION

The Etest system has obvious advantages over conventional methods of MIC determinations, especially in terms of its simplicity for laboratories that are not set up to do conventional MICs on a routine basis. For the species tested here, the Etest system has also shown itself flexible in terms of permitting some variation in inoculum size, temperature of incubation, and time of reading without significantly altering the results. Etest readings for *B. anthracis* in this study compared favorably with agar dilution readings in a subsidiary study set up to test this method (Table 4) and with studies reported previously (Table 6). In addition to extending the available data on *B. anthracis*, the results also expand the limited MIC data available for *B. cereus* from health-related sources (Table 5) (1, 28, 35).

One of the problems in this study, as with the majority of the other studies cited, is precisely defining the term "strain" and knowing for certain that cultures are unrelated. This issue becomes relevant when concluding that a particular proportion of the cultures exhibit a trait such as resistance to penicillin. Coker et al. (9) selected the set of 24 cultures they used, which were also included in this study, on the basis of the AFLP typing system (19), the best system available at present for differentiating *B. anthracis* isolates into strains. The remainder of the isolates used in the present study had not been typed by this method. Of the 76 total cultures included, 9 definitely had common ancestors and a further 8 may also have common ancestors. The penicillin-resistant group comprised of ASC 32, ASC 70, and ASC 183 is an example. ASC 32 and ASC 70 were believed to have been derived from the same patient, reaching the culture collection by different routes at different times, but differences in their antibiotic profiles were noted previously (22) (ASC 183 was a derivative of ASC 32 which had been cured of plasmid pXO1 [Table 1]). Less determinable is the relatedness or lack of it among, for example, wildlife isolates from Namibia. Differentiating strains of *B. cereus* and *B. thuringiensis* is somewhat easier through flagellar antigen-based serotyping systems, although isolates that cannot be serotyped are encountered quite frequently (Table 2).

Cavallo et al. (4) recorded a surprisingly high proportion (11.5%) of penicillin-resistant isolates of *B. anthracis* in their series, but they did not give their histories, apart from stating that 67 (70%) were isolated from environmental sources. It is possible that some of the isolates were related and that this may account for this high percentage of penicillin-resistant isolates.

The ability of *B. anthracis* to produce penicillinase was in fact recognized over half a century ago (3). Lightfoot et al. (22) demonstrated inducible β-lactamase production in a number of strains following exposure to a subinhibitory level of flucloxacillin. Inducible β-lactamases were again noted in relation to the anthrax events in the United States (6). The latter events led to published statements that penicillins, at least alone, are not recommended for the treatment of anthrax (5, 6). The *B. anthracis* genome sequence shows that this organism encodes two β-lactamases, a penicillinase and a cephalosporinase (6, 7, 25, 31). These β-lactamases are two examples of a significant number of genes (including those for motility, for example) that are shared with the closely related *B. cereus* which, though present, are not expressed as a result of a truncation in the *plcR* positive regulator gene (31, 34). However, the reality is that reports of naturally occurring resistance to penicillin in fresh clinical isolates are exceedingly rare and appear to number just five cases (2, 30), not all of which were well substantiated with further studies.

In this context, reports (4, 9; the latter not wholly confirmed here) that 11.5 and 12% of strains are resistant to penicillin are a little disturbing. Penicillin has long stood the test of time as the first choice for the treatment of anthrax in most parts of the world, and from the standpoint of the treatment of naturally acquired anthrax (as opposed to considerations relating to possible bioaggressive events), as it is cheap and readily available almost everywhere, it has to at least remain the basis of treatment schedules in animals and humans in developing countries. This view has been reinforced recently by others (32). Probably the fundamental principle, first stated half a century ago (14), is that adequate doses should be administered when penicillin is being used for treatment. It should be stressed, though, that there is no question that it is reasonable to add a second drug, where it is possible to do so, in cases showing signs of systemic involvement (32) or in other extreme situations such as known deliberate release exposures. That is by no means a new idea; the synergistic action of penicillin and streptomycin was recognized 40 years ago, and the recommendation was made then that both antibiotics be used at the same time in the treatment of septicemic anthrax (23).

The development of reduced susceptibility of *B. anthracis* to the quinolone ofloxacin but not to doxycycline following sequential subculture in subinhibitory concentrations has been demonstrated (8). The relatively low proportion of *B. anthracis* strains fully susceptible to erythromycin (15%) was somewhat surprising in view of the fact that this drug was regarded from the earliest days of antimicrobial chemotherapy (14) as an effective alternative to penicillin and is usually listed as such in medical microbiology texts.

B. cereus has long been associated with both food-borne illness and nongastrointestinal infections (11, 21, 24, 28, 33, 35). The latter infections are usually, but not always, opportunistic and are sometimes severe or life threatening. The incrimination of *B. thuringiensis* in infections is rare but has occurred, while *B. mycoides* appears to be totally nonpathogenic. From the many case reports of *B. cereus* infections, the broad picture is one of resistance to penicillin, ampicillin, cephalosporins, and trimethoprim and susceptibility to clindamycin, erythromycin, chloramphenicol, vancomycin, the aminoglycosides, and, usually, tetracycline. Ciprofloxacin was used successfully in the treatment of *B. cereus* wound infections (21). In a comparison of MIC methods, Andrews and Wise (1) found that, of five *B. cereus* strains, all were susceptible to ciprofloxacin and, with some variation between methods, to doxycycline; all were resistant to penicillin while, to tetracycline, two were susceptible, one was resistant, and two gave variable readings.

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